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Neuregulin-1 Enhances Depolarization-Induced GABA Release

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SUMMARY

Neuregulin-1 (NRG1), a regulator of neural development, has been shown to regulate neurotransmission at excitatory synapses. Although ErbB4, a key NRG1 receptor, is expressed in glutamic acid decarboxylase (GAD)-positive neurons, little is known about its role in GABAergic transmission. We show that ErbB4 is localized at GABAergic terminals of the prefrontal cortex. Our data indicate a role of NRG1, both endogenous and exogenous, in regulation of GABAergic transmission. This effect was blocked by inhibition or mutation of ErbB4, suggesting the involvement of ErbB4. Together, these results indicate that NRG1 regulates GABAergic transmission via presynaptic ErbB4 receptors, identifying a novel function of NRG1. Because both *NRG1* and *ErbB4* have emerged as susceptibility genes of schizophrenia, these observations may suggest a mechanism for abnormal GABAergic neurotransmission in this disorder.

INTRODUCTION

Neuregulin-1 (NRG1), a family of polypeptides that plays an important role in neural development, is implicated in nerve cell differentiation, neuron migration, neurite outgrowth, and synapse formation (Buonanno and Fischbach, 2001; Corfas et al., 2004). NRG1 and its receptor ErbB tyrosine kinases are expressed not only in the developing nervous system, but also in adult brain. In the adult, ErbB receptors are concentrated at the postsynaptic density (PSD), presumably via interaction with PDZ domain-containing proteins including PSD-95 and erbin (Garcia et al., 2000; Huang et al., 2000, 2001; Ma et al., 2003). NRG1 suppresses induction of LTP at Schaffer collateral-CA1 synapses in the hippocampus without affecting basal synaptic transmission (Huang et al., 2000; Ma et al., 2003). Subsequently, NRG1 was shown to reverse LTP and reduce whole-cell NMDA receptor currents in pyramidal neurons of prefrontal cortex, and was also shown to decrease NMDA receptor-mediated EPSCs in prefrontal cortex slices (Gu et al., 2005; Kwon et al., 2005). Interestingly, the *NRG1* gene is strongly associated with schizophrenia in diverse populations in Iceland, Scotland, China, Japan, and Korea (Fukui et al., 2006; Kim et al., 2006; Stefansson et al., 2002, 2003; Yang et al., 2003).

ErbB4 mRNA is enriched in regions where interneurons are clustered in adult brains (Lai and Lemke, 1991). GAD-positive neurons from the embryonic hippocampus express ErbB4 (Huang et al., 2000). During development, loss of NRG1/ErbB4 signaling alters tangential migration of cortical interneurons, leading to a reduction in the number of GABAergic interneurons in the cortex (Anton et al., 2004; Flames et al., 2004). In adult mice, deletion of ErbB4 in the central nervous system (CNS) resulted in lower levels of spontaneous motor activity, reduced grip strength, and altered cue use in performing a maze task (Golub et al., 2004). The *ErbB4* gene is also associated with schizophrenia (Law et al., 2006; Nicodemus et al., 2006).

γ-Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the mammalian forebrain. GABAergic inhibitory interneurons are essential to the proper functioning of the CNS (McBain and Fisahn, 2001). GABAergic dysfunction is implicated in several neurological disorders, including Huntington's chorea, Parkinson's disease, and epilepsy, and in psychiatric disorders such as anxiety, depression, and schizophrenia (Coyle, 2004).

This study investigates the role of NRG1 in GABAergic neurotransmission. We find that ErbB4 is expressed in GABAergic presynaptic terminals in the cerebral cortex. Treatment with NRG1 had no effect on basal GABA release, but it increased evoked release in cortical slices in a manner dependent on ErbB4. These observations identify a novel function of NRG1 and may suggest a mechanism

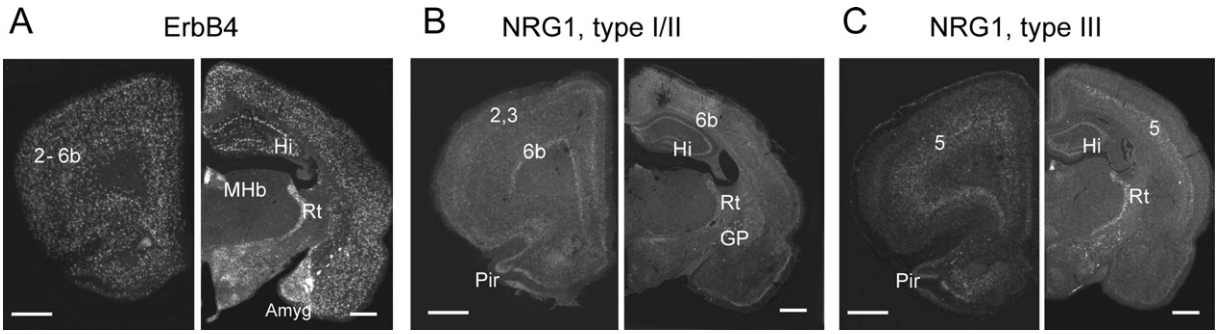

Figure 1. NRG1 and ErbB4 Are Expressed throughout Cortical Layers

(A–C) In situ hybridization in adult rat brain coronal sections using radiolabeled antisense RNA probes. (A) ErbB4-specific hybridization was detected in scattered cells throughout the cortex (layers 2–6b) in the rostral forebrain (left panel). Prominent hybridization is observed in scattered cells throughout the cortex and hippocampus (Hi), in the medial habenula (MHb), in the reticular nucleus of the thalamus (Rt), and in the intercalated masses of the amygdala (Amyg) in a more caudal section (right panel). Scale bar, 1 mm. (B) In the rostral forebrain (left panel), NRG1 type I/II-specific hybridization was detected in layers 2-3 and 6b of the cortex and in the piriform cortex (Pir). In caudal sections (right panel), NRG1 type I/II transcripts were detected in cortical layer 6b, in the reticular nucleus of the thalamus (Rt), in all fields of the hippocampus (Hi), and in scattered large cells in the globus pallidus (GP). (C) In the rostral forebrain (left panel), NRG1 type III-specific hybridization was detected in cortical layer 5 and in the piriform cortex (Pir). In more caudal sections (right panel), it was present in cortical layer 5, the reticular nucleus of the thalamus (Rt), and all fields of hippocampus (Hi).

for abnormal GABAergic neurotransmission in schizophrenia and epilepsy.

RESULTS

Localization of ErbB4 in GABAergic Presynaptic Terminals

ErbB4 transcripts were expressed throughout cortical layers 2–6b (Figure 1A) (Lai and Lemke, 1991; Yau et al., 2003). In addition, ErbB4 transcripts were identified at high levels in the medial habenula, the reticular nucleus of the thalamus, and in the intercalated masses of the amygdala. These observations are consistent with the notion that ErbB4 is expressed in interneurons. In further agreement, ErbB4 was shown to be present in GAD-positive neurons isolated from the hippocampus (Huang et al., 2000). To determine the in vivo subcellular localization of ErbB4 in GAD-positive neurons, we stained prefrontal sections of GFP-expressing inhibitory neurons (GIN) mice that express GFP under the control of the *gad1* promoter that directs specific expression in GABA interneurons, especially those that are somatostatin positive, in the hippocampus (Oliva et al., 2000). Presynaptic terminals of GABAergic neurons appear as discrete puncta-rings in the prefrontal cortex, surrounding the soma of postsynaptic neurons in cortical layers 2–6 (Figure 2A, arrows) (Pillai-Nair et al., 2005). The anti-ErbB4 antibody 0618 (Zhu et al., 1995) and sc-283 specifically recognized ErbB4 because their immunoreactivity was diminished in ErbB4 mutant mice (Figures 2G and 2H). As shown in Figure 2A, ErbB4 was detected in puncta-rings and neuropils, colocalizing with GFP. Quantitatively, about 90% of puncta-rings and neuropils in the prefrontal cortex expressed ErbB4 (Figure 2B). These results suggest that ErbB4 is present at terminals of GABAergic neurons, including somatostatin neurons. To test this hypothesis further, we determined whether ErbB4 colocalizes with GAD65 and vesicular GABA transporter (VGAT), both well-characterized markers of GABAergic terminals (Tafoya et al., 2006). The ErbB4 immunoreactivity colocalized with GAD65 and VGAT in puncta-ring-like structures (Figures 2C and 2D). Twenty-three percent of GAD65 clusters and forty-seven percent of VGAT clusters were ErbB4-positive, suggesting ErbB4 localization at specific subsets of GABA terminals (Figures 2E and 2F). On the other hand, 26% and 70% of ErbB4 clusters colocalized with GAD65 and VGAT, respectively, in agreement with the notion that ErbB4 is also localized at non-GABAergic synapses (Huang et al., 2000). Taken together, these results indicate that ErbB4 is present at groups of presynaptic terminals of GABAergic neurons in the cerebral cortex.

Increase in Depolarization-Evoked GABA Release by NRG1

The presynaptic localization of ErbB4 in GABAergic neurons suggested to us that NRG1 may regulate GABAergic neurotransmission. To test this hypothesis, we determined effects of NRG1 on GABA release in cortical slices by both biochemical and electrophysiological approaches. Basal [^{3}H]GABA release was low, at a rate of 3.75% ± 0.35% (n = 8) of total radioactivity per 10 min (Figure 3A). Treatment of slices with 20 mM KCl, a treatment known to depolarize neurons, increased [^{3}H]GABA release by 2.5- to 3.5-fold within 10 min (Figure 3A). NRG1 had no effect on basal [^{3}H]GABA release; by contrast, it increased depolarization-evoked GABA release in a dose-dependent manner (Figures 3A and 3B and Figure S1A in the Supplemental Data). This effect was not inhibited by antagonists of glutamate receptors, suggesting that the increase in GABA release does not require glutamatergic signaling (Figure S1B). To demonstrate that NRG1 regulates the physiological function of GABA transmission, inhibitory

postsynaptic currents (IPSCs) were recorded from prefrontal cortical slices. As shown in Figures 3C–3F, NRG1 did not appear to affect the frequency, amplitude, and decay times of miniature IPSCs (mIPSCs) that were blockable by bicuculline, a GABA-A receptor antagonist (Figure S1C and data not shown). These results are in agreement with observations above that basal GABA release was not affected. By contrast, as shown in Figure 3G, it enhanced evoked IPSCs (eIPSCs) that were sensitive to bicuculline (Figure S1D). The increase in eIPSCs had a similar dose-response curve to that of evoked [^{3}H]GABA release (Figure 3H) and was abolished when NRG1 was heat-denatured (Figure 3I). Furthermore, the NRG1 regulation remained unchanged in the presence of antagonists of metabotropic glutamate receptors, cholinergic receptors, serotonin receptors, adrenergic receptors, dopamine receptors, or some combination thereof (Figure S1E). As a control, BDNF decreased depolarization-evoked GABA release and eIPSCs in cortical slices, in agreement with earlier studies (Canas et al., 2004; Frerking et al., 1998). These results indicate that NRG1 increases evoked GABA release without affecting basal release, likely via a direct effect on GABAergic presynaptic terminals.

NRG1 Effects on GABAergic Presynaptic Terminals

To further determine whether NRG1 regulates GABA release directly at presynaptic terminals, we performed the following two experiments. First, we investigated whether NRG1 is able to regulate [^{3}H]GABA release from synaptosomes in the absence of their neural circuit. As shown in Figure 4A, NRG1 increased depolarization-evoked GABA release from synaptosomes while having no effect on basal GABA release. Moreover, this effect was concentration-dependent, with a maximal response of 28% ± 1.5% (n = 6), similar to that observed in cortical slices (Figure 4A). Second, we characterized the paired-pulse ratios (PPRs) of control and NRG1-affected eIPSCs in response to two stimulations. At inhibitory synapses, a second stimulation generates a smaller eIPSC because of depletion of vesicles in the releasable pool by the first stimulation (Lambert and Wilson, 1994). Figure 4B (left panel) shows averaged traces of eight consecutive eIPSCs induced by paired stimuli at different interpulse intervals. The PPRs at 25 ms intervals were reduced from 0.86 ± 0.07 in control to 0.68 ± 0.05 in NRG1-treated slices ($n = 6$, $p < 0.01$). The reduction in PPRs remained even at 200 ms intervals. The depression effect of NRG1 on the amplitudes of the second eIPSCs provides further evidence that NRG1 regulates evoked GABA release by a presynaptic mechanism. In addition, these results also suggest that NRG1 may increase the probability of GABA release in response to depolarization.

Endogenous NRG1 Is Necessary to Maintain Activity-Dependent GABA Release

NRG1 is expressed in various regions in the brain (Law et al., 2004). NRG1 type I/II transcripts were detected prominently in cortical layer 6b and at lower levels in layers 2–3 (Figure 1B). In comparison, NRG1 type III transcripts were primarily detected in cortical layer 5 (Figure 1C). Hybridization of NRG1 type I/II was also observed in the reticular nucleus of the thalamus and in cholinergic interneurons in the globus pallidus. NRG1 type III was expressed in the reticular nucleus of the thalamus. Both NRG1 isoforms were also observed in the piriform cortex and throughout the hippocampus. Notably, the distinct isoforms of NRG1 appear to be expressed in a laminar-specific and largely nonoverlapping manner in the cortex. These observations indicate that NRG1 is available in various areas in the brain including the cerebral cortex. To determine whether endogenous NRG1 regulates GABA release, we generated ecto-ErbB4, which contains the entire extracellular region of ErbB4 fused to an FC fragment. Ecto-ErbB4 binds to and thus prevents NRG1 from interacting with ErbB receptor kinases. As shown in Figure 5A and Figure 5B, treatment with ecto-ErbB4 inhibited NRG1 activation of ErbB4 in GAD-positive neurons (see Figure S2 for characterization of the anti-phospho-ErbB4 antibody). Such treatment blocked NRG1 potentiation of eIPSCs in a dose-dependent manner (Figures 5C and 5D), demonstrating the neutralizing ability of ecto-ErbB4. NRG1-enhanced evoked GABA release was also inhibited by ecto-ErbB4 (Figure 5D). Remarkably, treatment with ecto-ErbB4 alone reduced both evoked GABA release and eIPSCs in the absence of exogenous NRG1 (Figure 5D). These observations and results from studies of inhibitors of ErbB4 suggest a role for endogenous NRG1 in regulating evoked GABA release.

ErbB4 Is Necessary for NRG1 Enhancement of Evoked GABA Release

Of the three ErbB kinases, ErbB2 and ErbB4, but not ErbB3, are catalytically active (Citri and Yarden, 2006). To determine which ErbB is involved in NRG1 regulation of evoked GABA release, cortical neurons were treated with AG879 and AG1478, specific inhibitors of ErbB2 and ErbB4, respectively (Fukazawa et al., 2003). ErbB4 tyrosine phosphorylation in response to NRG1 was blocked in neurons pretreated with AG1478, but not AG879 (Figures 6A and 6B). Treatment with AG1478 prevented NRG1 from increasing evoked GABA release and increasing amplitude of eIPSCs in cortical slices (Figure 6C). These results suggest a role of ErbB4 in NRG1 regulation of GABAergic transmission. As observed with ecto-ErbB4, AG1478 alone decreased depolarization-evoked [^{3}H]GABA release and the amplitude of eIPSCs (Figure 6C), providing further evidence that endogenous NRG1 activity may be necessary to maintain GABA release elicited by neuronal activation. In a control experiment, treatment with AG879 had no detectable effect on evoked GABA release and the amplitude of eIPSCs in the presence or absence of exogenous NRG1 (Figure 6C). Taken together, these observations demonstrate that activation of ErbB4, but not ErbB2, is required for NRG1's effect.

To investigate the involvement of ErbB4 further, we characterized evoked GABA release in ErbB4 mutant mice. ErbB4 null mutant mice die around E11. The embryonic

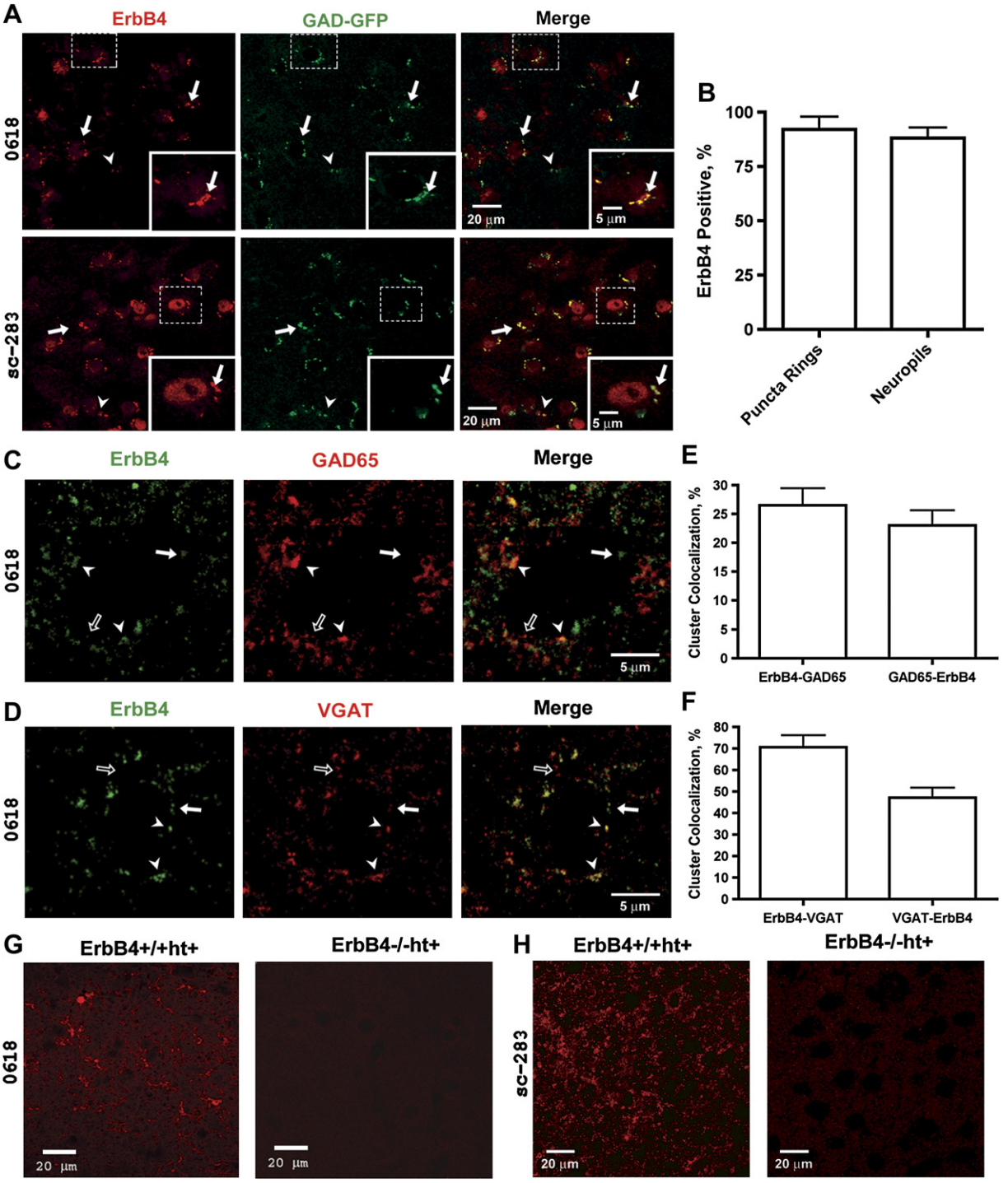

Figure 2. ErbB4 Is Present at Presynaptic Terminals of GABAergic Neurons

(A) Coronal sections of prefrontal cortex of GIN-GFP mice were stained with anti-ErbB4 antibody 0618 (top panels) or with the antibody sc-283 (bottom panels). Immunoactivity was visualized by Alexa 594-conjugated secondary antibody. GAD-positive terminals (expressing GFP) were visualized by excitation at 488 nm. Arrows, GFP-positive puncta-ring structures surrounding pyramidal neurons; arrowheads, neuropils; inset, enlarged areas.

(B) Quantitative analysis of puncta-rings and neuropils that are positive for ErbB4. The antibody used for quantification was 0618. Shown are means ± SEM; n = 60 for puncta-rings and n = 10 for neuropils of 20 independent sections.

(C and D) Coronal sections of prefrontal cortex were stained with anti-ErbB4 antibody 0618 and anti-GAD65 (G1166) and anti-VGAT (131003) antibodies. Immunoactivity was visualized by Alexa 488- and Alexa 594-conjugated secondary antibodies, respectively. Arrowheads, colocalization of ErbB4 and GAD65 or VGAT; arrows, ErbB4-positive alone; hallow arrows, GAD65- or VGAT-positive alone.

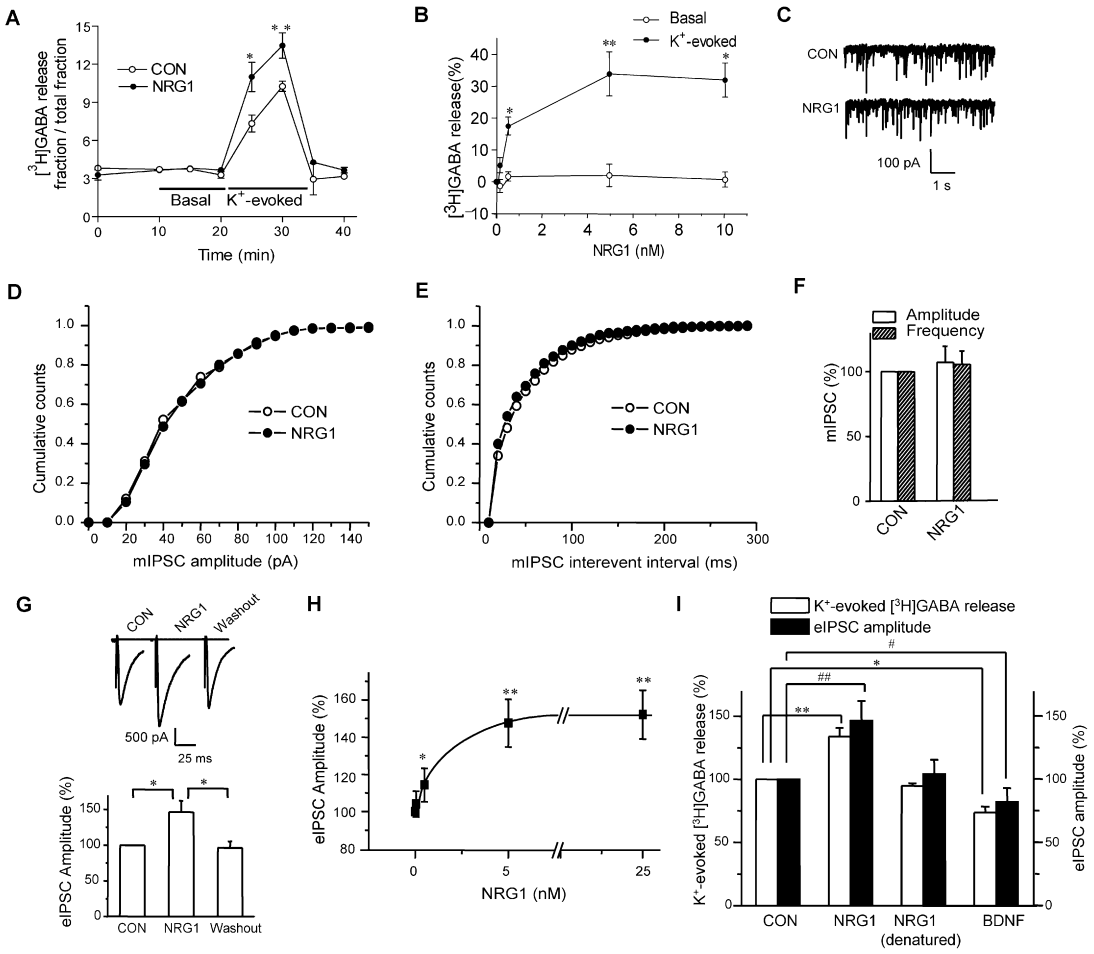

Figure 3. NRG1 Increases Depolarization-Evoked [^{3}H]GABA Release and eIPSC Amplitude

(A) Cortical slices were preloaded with [^{3}H]GABA for 30 min in the presence of β-alanine (1 mM), an inhibitor of [^{3}H]GABA uptake by glial cells, aminooxyacetic acid (0.1 mM), an inhibitor of GABA degradation, and nipecotic acid (1 mM), an inhibitor of the GABA transporter in neurons. Basal and depolarization (20 mM KCl)-evoked release of [^{3}H]GABA were monitored sequentially. The sum of the basal release, depolarization-evoked release, and the residual [^{3}H]GABA was taken as 100%. In comparison with controls (open circles), NRG1 (closed circles) had no effect on basal [^{3}H]GABA release, but increased depolarization-evoked [^{3}H]GABA release.

(B) Dose-dependent potentiation of evoked [^{3}H]GABA release. Raw data of a representative experiment are presented in Figure S1A.

(C) Representative traces of mIPSCs in pyramidal neurons in prefrontal cortical slices.

(D) Cumulative plots of mIPSC amplitudes.

(E) Cumulative plots of mIPSC frequencies.

(F) No effect of NRG1 on mIPSCs in pyramidal neurons in prefrontal cortical slices (n = 12).

(G) Increased eIPSCs in NRG1-treated slices. (Top) Representative eIPSCs of control, NRG1-treated, or NRG1-treated/washed slices. (Bottom) Quantitative analysis of eIPSC amplitudes. n = 12, *p < 0.01.

(H) Dose-dependent effect of NRG1 on eIPSCs. n = 6, *p < 0.05, **p < 0.01.

(I) Denatured NRG1 failed to increase depolarization-evoked [^{3}H]GABA release and eIPSC amplitude. n = 8 for [^{3}H]GABA release; for eIPSCs, n = 6 for control, NRG1, and denatured NRG1, and n = 4 for BDNF. *p < 0.05, $^{\#}$p < 0.05; **p < 0.05, $^{\#\#}$p < 0.01.

lethality can be genetically rescued by expressing ErbB4 under a cardiac-specific myosin promoter (Tidcombe et al., 2003). This line of mice (*ErbB4*$^{-/-}$*ht*$^{+}$), however, does not express ErbB4 in the brain (Figures 7A and 7B) or other noncardiac tissues (data not shown). Ablation of the ErbB4 gene had no effect on basal and depolarization-evoked [^{3}H]GABA release (Figure 7C). However, unlike in control slices, NRG1 was unable to increase evoked

(E and F) Quantitative analysis of ErbB4 clusters with GAD65 and with VGAT, and VGAT and GAD65 clusters with ErbB4. More than 1100 clusters of five independent sections were scored. Shown are means ± SEM.

(G and H) Specificity characterization of anti-ErbB4 antibodies. Coronal sections of prefrontal cortex of *ErbB4*$^{+/+}$*ht*$^{+}$ and *ErbB4*$^{-/-}$*ht*$^{+}$ mice were incubated with the anti-ErbB4 antibodies 0618 and sc-283. Immunoactivity was visualized by Alexa-conjugated secondary antibodies.

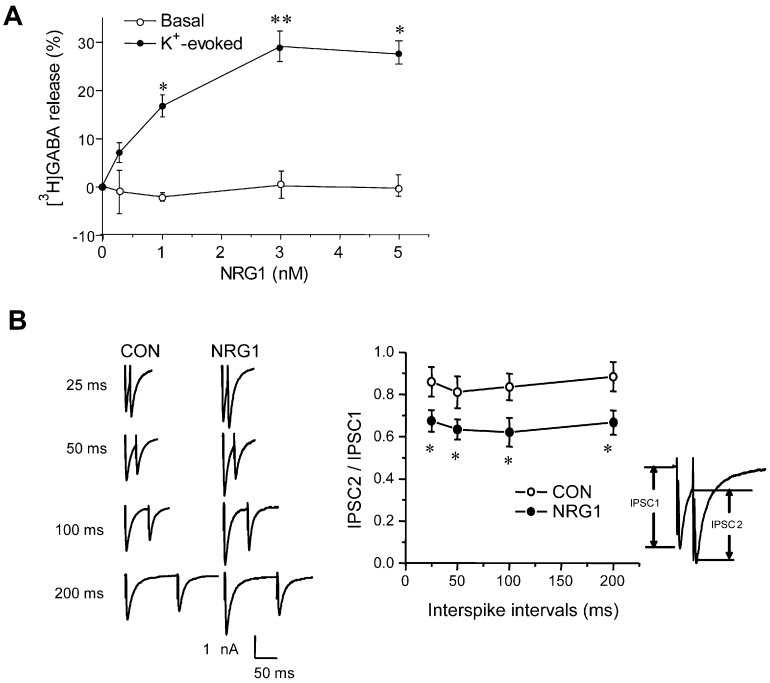

Figure 4. Effects of NRG1 on Presynaptic Terminals

(A) NRG1 increases depolarization-evoked [^{3}H]GABA release from synaptosomes. [^{3}H]GABA-loaded cortical synaptosomes were treated with 5 nM NRG1 with (evoked) or without (basal) 20 mM KCl. [^{3}H]GABA release was assayed 10 min after NRG1 stimulation. Shown are means ± SEM of six individual experiments in triplicate. *$p < 0.05$, **$p < 0.01$.

(B) NRG1 reduces PPRs of GABAergic transmission in the prefrontal cortex. (Left) Averaged traces of eight consecutive recordings induced by paired stimuli (10 s apart) separated by indicated interpulse intervals. (Right) PPRs as a function of interpulse intervals. The amplitudes of the first and second IPSCs were measured as indicated in the inset. n = 6, *$p < 0.05$.

[^{3}H]GABA release and eIPSC amplitude in *ErbB4*$^{-/-}$*ht*$^{+}$ slices (Figures 7C and 7D). These observations identify an important role of ErbB4 in NRG1 regulation of evoked GABA release.

DISCUSSION

The major findings of this study are as follows. First, ErbB4, a receptor for NRG1, is present in GABAergic terminals of the prefrontal cortex. Second, NRG1 facilitates evoked release of GABA from slices of the prefrontal cortex, but has no effect on basal GABA release. Third, the potentiation effect of NRG1 must require ErbB4 because it was blocked by the ErbB4 inhibitor AG1478 and was abolished in cortical slices of ErbB4 mutant mice. In addition, we provided evidence that evoked GABA release and eIPSCs in the absence of exogenous NRG1 were blocked by inhibitors of NRG1 signaling, suggesting a role of endogenous NRG1 in regulating GABA neurotransmission. Together, these results identify a novel function of NRG1—regulation of GABAergic transmission via presynaptic ErbB4 receptors. These results suggest that NRG1 may regulate the activity of cortical interneurons, providing insight into potential mechanisms by which this trophic factor regulates synaptic plasticity and pathogenesis of schizophrenia and epilepsy.

NRG1 and Neurotransmission at Excitatory and Inhibitory Synapses

NRG1 has been shown to regulate differentiation of neural cells, neuronal navigation, and neuron survival in developing CNS (Buonanno and Fischbach, 2001; Corfas et al., 2004). In the peripheral nervous system, NRG1 signaling is implicated in Schwann cell differentiation and myelination, muscle spindle development, and synapse-specific expression of AChR subunit genes (Adlkofer and Lai, 2000; Fischbach and Rosen, 1997; Hippenmeyer et al., 2002; Si et al., 1996). Interestingly, NRG1 and its receptor ErbB kinases are continuously expressed in various brain regions, including the prefrontal cortex, hippocampus, cerebellum, oculomotor nucleus, superior colliculus, red nucleus, substantia nigra, and pars compacta (Lai and Lemke, 1991; Law et al., 2004; Yau et al., 2003). Moreover, ErbB4 colocalizes with PSD-95 and NMDA receptors in hippocampal neurons (Garcia et al., 2000; Huang et al., 2000). Furthermore, NRG1 signaling may be increased by the interaction of ErbB4 with PSD-95 (Huang et al., 2000). These observations suggest that NRG1 may play a role in synaptic plasticity, maintenance or regulation of synaptic structure, or some combination thereof in adult brain. Indeed, we found that NRG1 blocks induction of long-term potentiation (LTP) at Schaffer collateral-CA1 synapses (Huang et al., 2000). NRG1 can depotentiate LTP at hippocampal CA1 synapses and reduce whole-cell NMDA receptor, but not AMPA receptor, currents in prefrontal cortex pyramidal neurons (Gu et al., 2005; Kwon et al., 2005). Recently, ErbB4 has been shown to play a key role in activity-dependent maturation and plasticity of excitatory synaptic structure and function (Li et al., 2007).

This study provides evidence that ErbB4 is present at GABAergic terminals in the prefrontal cortex. The identification of the subtype or subtypes of GABA interneurons that express ErbB4 will require further investigation. Interestingly, ErbB4 colocalizes with GAD-GFP in GIN mice. An earlier study demonstrated that hippocampal

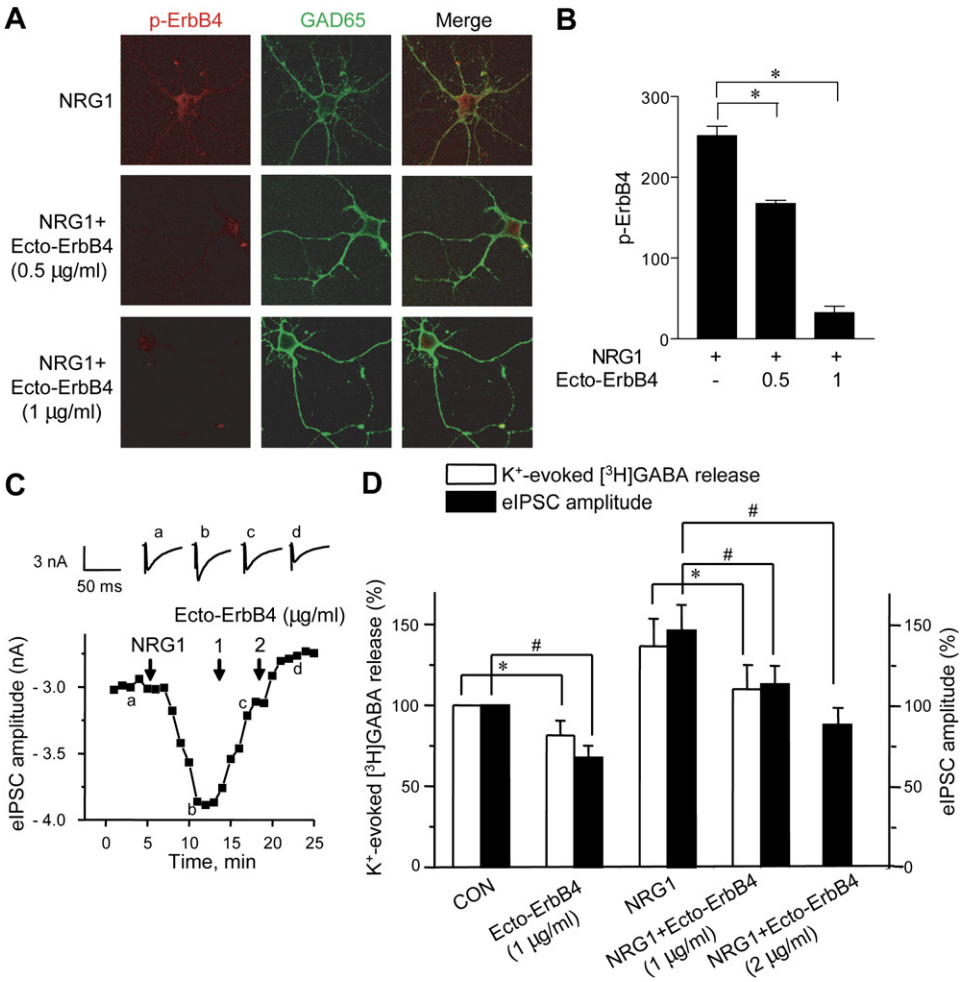

Figure 5. Suppression of NRG1-Enhanced GABA Release by Ecto-ErbB4

(A) Ecto-ErbB4 inhibition of NRG1 activation of ErbB4 in GAD65-positive cortical neurons. Cortical neurons were pretreated with ecto-ErbB4 for 10 min prior to the addition of NRG1 (5 nM, final concentration) for another 10 min, fixed, and stained with anti-p-ErbB4 and anti-GAD65 antibodies that were visualized with Alexa 594 and FITC-coupled secondary antibodies, respectively. Scale bar, 20 μm.

(B) Quantitative analysis of data in (A). Images were captured with a Zeiss LSM confocal microscope and analyzed by Image J software (NIH). Ecto-ErbB4 treatment inhibits NRG1-induced ErbB4 phosphorylation. n = 7, *$p < 0.05$.

(C) Ecto-ErbB4 inhibition of eIPSCs. Cortical slices were treated with sequential addition of NRG1 (5 nM) and ecto-ErbB4 (1 μg/ml and 2 μg/ml) (all final concentrations). eIPSCs were recorded as in Figure 3G. Shown are data from a representative experiment. On the top are averaged traces before (a) and after (b) NRG1, and after different dosages of ecto-ErbB4 ([c] and [d], 1 and 2 μg/ml, respectively).

(D) Inhibition by ecto-ErbB4 of depolarization-evoked GABA release and eIPSCs. Cortical slices were treated with 1 or 2 μg/ml ecto-ErbB4 for 10 min prior to assays of [^{3}H]GABA and eIPSCs. n = 5 for [^{3}H]GABA release, n = 6 for eIPSCs. *$p < 0.01$ and $^{\#}p < 0.01$ for [^{3}H]GABA release and eIPSCs, respectively.

GAD-GFP-labeled neurons of these mice are mostly somatostatin positive (Oliva et al., 2000). Whether GFP-labeled neurons in the prefrontal cortex are somatostatin positive was not characterized in detail. Nevertheless, we found that NRG1 activates ErbB4 and regulates GABAergic transmission. This trophic factor has no effect on basal GABA release but increases GABA release evoked by neuronal activation. More work is needed to determine whether NRG1 regulates neurotransmission of other GABAergic neurons. Because glutamatergic neurotransmission can be regulated by NRG1 (Gu et al., 2005; Li et al., 2007) and because glutamatergic activity is known to increase GABAergic transmission (Belan and Kostyuk, 2002), it is possible that NRG1 regulation of evoked GABA release may be mediated by a glutamatergic mechanism. Our results, however, suggest otherwise; NRG1 enhancement of evoked [^{3}H]GABA release was not attenuated by inhibitors of NMDA and AMPA receptors. Moreover, NRG1 enhanced eIPSCs in the presence of these inhibitors. Therefore, we propose that NRG1 regulates GABA release by directly activating ErbB4 receptors on presynaptic terminals. The presence of ErbB4 in

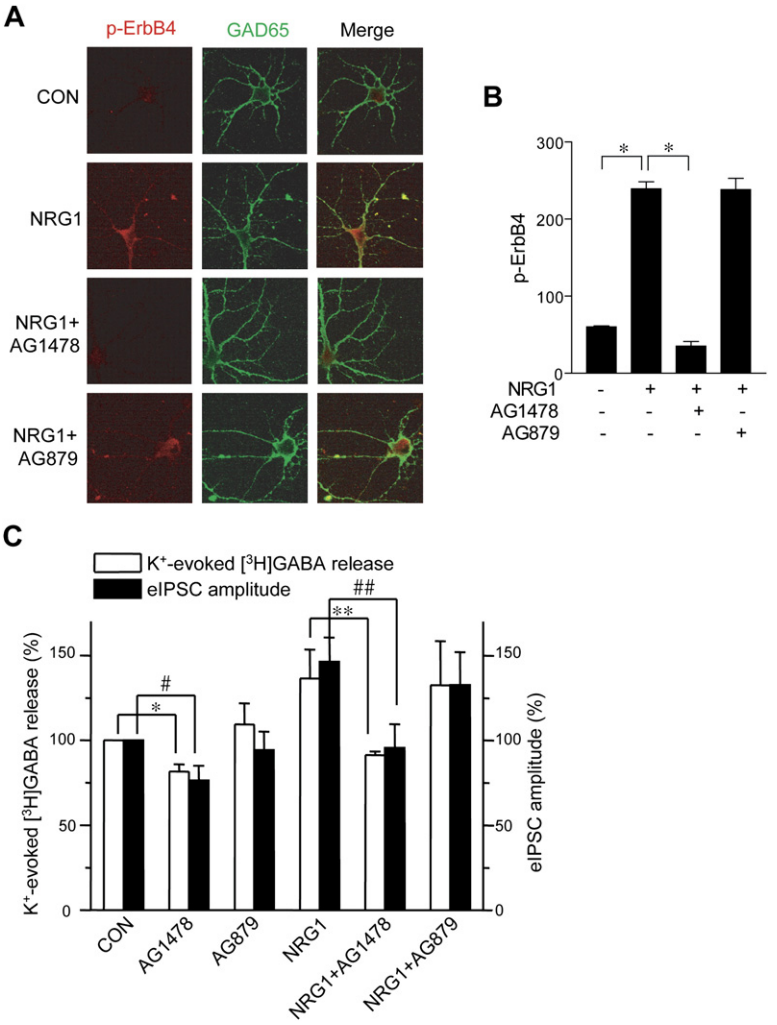

Figure 6. Inhibition of ErbB4 Blocks NRG1 Potentiation of GABA Release

(A) NRG1 activation of ErbB4 was inhibited by AG1478, but not AG879. Cortical neurons were treated with 5 μM AG1478, an inhibitor of ErbB4, or AG879, an inhibitor of ErbB2, for 10 min prior to the addition of NRG1 (5 nM, final concentration). Neurons were fixed and stained with phospho-ErbB4 and GAD65 antibodies, and visualized with Alexa 594 and FITC-coupled secondary antibodies, respectively. Scale bar, 20 μm.

(B) Quantitative analysis of data in (A). n = 8, $^{*}p < 0.05$.

(C) Inhibition of NRG1 enhancement of evoked GABA release by AG1478. Cortical slices were treated with 5 μM AG1478 or AG879 for 10 min prior to assays of [^{3}H]GABA or eIPSC recording. n = 5 for [^{3}H]GABA release, n = 6 for eIPSCs. $^{*}p < 0.05$, $^{\#}p < 0.05$; $^{**}p < 0.01$, $^{\#\#}p < 0.01$.

GAD-GFP-positive puncta-ring-like structures and the colocalization with GAD65 and VGAT provide anatomical evidence in support of this notion. Moreover, NRG1 was able to increase depolarization-evoked GABA release from synaptosomes that were free of interneural network, suggesting that the regulatory machinery for NRG1 was present in presynaptic terminals. Furthermore, NRG1 decreases PPRs of eIPSCs in response to two consecutive stimulations, suggesting that it may facilitate vesicle release evoked by neuronal activation of interneurons.

NRG1, ErbB4, and Neurological and Psychiatric Disorders

Schizophrenia exhibits familial characteristics, which suggests a strong genetic component. Disturbances in GABAergic neurotransmission have been thought to be a pathologic mechanism of schizophrenia. Postmortem studies of patient brains reveal decreased levels of the mRNA encoding GAD67 (Hashimoto et al., 2003) and the GABA transporter GAT-1 (Ohnuma et al., 1999). On the other hand, GABA-A receptor mRNA was shown to be increased in the prefrontal cortex (Ohnuma et al., 1999). Furthermore, treatment of schizophrenia with antiepileptic drugs that target GABAergic transmission has shown positive results (Hosak and Libiger, 2002).

This paper provides evidence that endogenous NRG1 plays a role in maintaining evoked GABA release. First, treatment with ecto-ErbB4 alone attenuated evoked GABA release, presumably by neutralizing endogenous NRG1. Second, inhibition of ErbB4 reduced evoked GABA release in the absence of exogenous NRG1. In light of the fact that interneuron activity in vivo could be high (Mountcastle et al., 1969), it is likely that NRG1 plays an important role in controlling neuronal activity in the brain. These data are consistent with expression of NRG1 by cortical pyramidal neurons and ErbB4 by interneurons. While ErbB4 is expressed in interneurons throughout the cortex, distinct isoforms of NRG1 appear to be expressed in a lamina-specific and largely nonoverlapping manner in the cortex. The readily available NRG1 may maintain basal activity-dependent GABAergic transmission. Interestingly, NRG1 or ErbB4 heterozygotes show hyperactivity in an

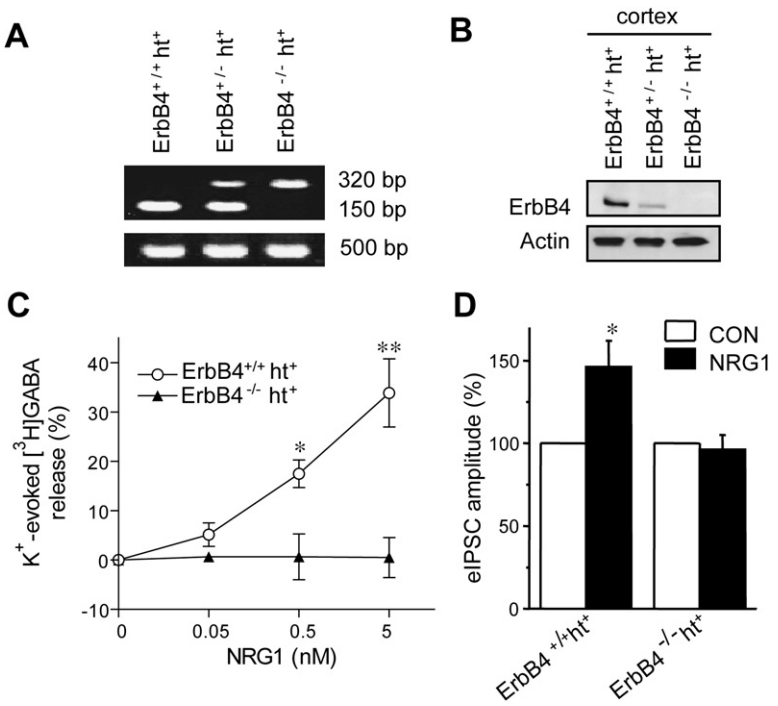

Figure 7. NRG1 Potentiation of GABA Release Was Diminished in ErbB4 Mutant Mice

(A) Genotyping of heart-rescued *ErbB4*$^{-/-}$ mice. Transgenic mice (ht^{+}) expressing ErbB4 under the control of the MHC promoter were crossed with *ErbB4*$^{+/-}$ mice to generate *ErbB4*$^{-/-}$;*ErbB4*^{HEART} (*ErbB4*$^{-/-}$*ht*$^{+}$) (Tidcombe et al., 2003). The ErbB4 wild-type allele yields ~150 bp, whereas the mutant allele yields ~320 bp. The heart rescue transgene yields ~500 bp.

(B) Western blots showing that ErbB4 was not expressed in the brains from *ErbB4*$^{-/-}$*ht*$^{+}$ mice. Equal loading was shown by immunoblotting for actin.

(C) NRG1 enhancement of depolarization-evoked GABA release was abolished in *ErbB4*$^{-/-}$*ht*$^{+}$ cortical slices. [^{3}H]GABA release was assayed as in Figure 3A. n = 6.

(D) NRG1 potentiation of eIPSCs was lost in ErbB4 mutant mice. Cortical slices of control (*ErbB4*$^{+/+}$*ht*$^{+}$) and *ErbB4*$^{-/-}$*ht*$^{+}$ mice were recorded for eIPSCs. Shown are normalized eIPSC amplitudes. n = 6, *p < 0.05. The eIPSC amplitudes in *ErbB4*$^{+/+}$*ht*$^{+}$ and *ErbB4*$^{-/-}$*ht*$^{+}$ were 1014 ± 170 and 598 ± 160 pA, respectively. n = 17, p < 0.01.

open field (Gerlai et al., 2000; Stefansson et al., 2002). Further investigation of NRG1's role in regulating GABA transmission could be useful in understanding the pathogenesis of schizophrenia and epilepsy.

EXPERIMENTAL PROCEDURES

Reagents and Animals

The NRG1 used is a recombinant polypeptide containing the entire EGF domain of the β-type NRG1 (rHRG β177–244) from Dr. Mark Sliwkowski (Holmes et al., 1992). It was prepared in 1% bovine serum albumin (BSA). BDNF was a gift from Regeneron Pharmaceuticals. The ectodomain of ErbB4 (aa 1–659, ecto-ErbB4) was subcloned into pC4DNA/Fc to generate pErbB4ex/Fc. Stable HEK293 cells expressing ecto-ErbB4 were generated and cultured in IgG-low medium for condition media collection. ErbB4ex/Fc was purified by a HiTrap column (Amersham). AG1478 and AG879 were from Calbiochem; poly-L-lysine, nipecotic acid, β-alanine and TMPH (2,2,6,6,-Tetramethylpiperidin-4-yl heptanoate) from Sigma; DL-AP5, CNQX, TTX, bicuculline, LY341495, ipratropium, nicergoline, sotalol, metergoline, MDL 72222, RS 23597-190, and L-741742 from Tocris Bioscience; and aminooxyacetic acid from Chemika. When necessary, chemicals were dissolved in dimethylsulfoxide (DMSO, Sigma); the final concentration of DMSO was 0.001% or less when applied to brain slices. Antibodies were from Sigma (GAD65, G1166); Cell Signaling Technology [ErbB4, #4795; p-ErbB4 (Y1284), #4757]; Transduction Labs (phosphotyrosine, 610024); NeoMarkers (ErbB2, MS-303-PO; ErbB3, MS-229-PO); Santa Cruz Biotechnology (ErbB4, sc-283); and Synaptic Systems (VGAT, 131003). *ErbB4*$^{-/-}$*ht*$^{+}$ mice were kindly provided by Martin Gassmann (Tidcombe et al., 2003). GAD-GFP mice were from the Jackson Lab.

[^{3}H]GABA Release

[^{3}H]GABA release from cerebral cortical slices was assayed as described previously (Woo et al., 2002). Briefly, male Sprague-Dawley rats (200–250 g) or *ErbB4*$^{+/+}$*ht*$^{+}$ and *ErbB4*$^{-/-}$*ht*$^{+}$ mice were decapitated; cerebral cortices were dissected out and sliced with a McIlwain tissue chopper. Slices (0.25 × 0.25 mm) were preincubated for 15 min at 37°C in 10 ml of oxygenated Krebs-HEPES buffer (KHB, pH 7.4) containing 25 mM HEPES-sodium salt, 100 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 10 mM glucose. Slices were incubated for 30 min with 50 nM [^{3}H]GABA (Perkin-Elmer Life Sciences, 33.7 Ci/mmol) in KHB containing 1 mM β-alanine to prevent [^{3}H]GABA uptake by glial cells. For basal GABA release, slices were incubated in KHB for 10 min, after which aliquots of the medium were collected. For depolarization-evoked GABA release, slices were incubated with KHB containing 20 mM KCl for 10 min in the presence or absence of NRG1 (5 nM, unless otherwise indicated). In some experiments, inhibitors or vehicle were added 10 min prior to NRG1 stimulation. Medium was collected and counted in scintillation solution by a β counter. Slices were incubated in 0.2 N HCl for 45 min to extract residual radioactivity. The sum of basal release, the release in the presence of KCl (evoked), and the residual [^{3}H]GABA was taken as 100%. Aminooxyacetic acid (0.1 mM), an inhibitor of GABA degradation, and nipecotic acid (1 mM), an inhibitor of the GABA transporter in neurons, were present in all solutions.

To measure GABA release from synaptosomes, cerebral cortex was isolated from adult rats and homogenized in 10 volumes of the homogenization buffer (0.32 M sucrose, 5 mM HEPES-NaOH [pH 7.4], and 1 mM EDTA) with glass-Teflon homogenizer (Turner and Goldin, 1989). Homogenates were cleared by low-speed centrifugation (1000 × g for 10 min) to remove nuclear fractions and cell debris. The supernatant was centrifuged at 14,500 × g for 20 min and the resulting synaptosomal pellet (P2) was resuspended in ice-cold oxygenated KHB buffer to 2 mg protein/ml. Synaptosomes were incubated at 37°C for 10 min before the addition of [^{3}H]GABA (33.7 Ci/mmol, 50 nM) in oxygenated KHB for 10 min. The loading reaction was stopped by a centrifugation at 12,000 × g for 1 min and the pellet resuspended to 1 mg protein/ml with ice-cold oxygenated KHB. To measure [^{3}H]GABA release, synaptosomes (50 μg protein in 100 μl) were stimulated without (basal) or with (evoked) 20 mM KCl at 37°C for 10 min and centrifuged at 12,000 × g for 1 min at 4°C. Aliquots of the supernatant and SDS-solubilized pellets were counted. The sum of the radioactivity in the supernatant and pellets was taken as 100%.

Electrophysiological Recordings in Slices

Transverse prefrontal cortical slices (0.3 mm) were prepared from P28–P36 mice using a Vibroslice (Leica VT 1000S) in the ice-cold solution, which contained 2.5 mM KCl, 1.25 mM NaH_2PO_4, 10 mM $MgSO_4$, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose, and 230 mM sucrose. Slices were allowed to recover for at least 2 hr in ACSF (1 hr at 34°C followed by 1 hr at 22°C) in a solution containing 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose. Slices were placed in the recording chamber and superfused (1.5 ml/min) with ACSF at 34°C. All solutions were saturated with 95% O_2/5% CO_2. Neurons were visualized with an IR-sensitive CCD camera with a 40× water-immersion lens (Zeiss, Axioskop2 Fsplus) and recorded using whole-cell voltage-clamp techniques (MultiClamp 700B Amplifier, Digidata 1320A analog-to-digital converter) and pClamp 9.2 software (Axon Instruments). Glass pipettes were filled with the solution containing 125 mM Cs-gluconate, 10 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES, 1 mM EGTA, 0.1 mM $CaCl_2$, 10 mM sodium phosphocreatine, 4 mM Mg-ATP, 0.3 mM GTP, 0.2 mM leupeptin, and 5 mM lidocaine N-ethylchloride (QX314) (pH 7.2, with the osmolarity adjusted to 280 mOsm with sucrose). The resistance of pipettes was 2–3 MΩ. For mIPSC recording, QX314 was omitted in the pipette filling solution, whereas 1 μM TTX was included in the superfusing solution. eIPSCs were generated with a two-concentric bipolar stimulating electrode (25 μm pole separation; FHC, ME) positioned ~100 μm from the neuron under recording. Single or paired pulses of 0.2 ms were delivered at 0.1 Hz and synchronized using a Mater-8 stimulator (A.M.P.I). The holding potential for both mIPSCs and eIPSCs was −65 mV. All experiments were done at 34°C in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and AP-5 (50 μM) to block AMPA/NMDA receptors. Data were collected when series resistance fluctuated within 15% of initial values (8–15 MΩ), they were filtered at 2 kHz, and they were sampled at 10 kHz.

Cell Culture

Primary cortical neurons were cultured as described previously (Huang et al., 2000). Briefly, cerebral cortex was dissected out of Sprague-Dawley rat embryos (E18) and dissociated by gentle trituration in PBS (Cellgro). Cells were seeded on poly-L-lysine-coated 12-well plates and cultured in Neurobasal media (Gibco). Experiments were performed 14 days after seeding (DIV14). C2C12 cells were obtained from E. S. Ralston (NIH) and cultured as previously described (Si et al., 1996). To generate ecto-ErbB4, HEK293 cells were cotransfected with pC4-B4Ex/Fc, which expresses the entire ectodomain fused with the Fc fragment, and pEGFP-C1, which contains the neomycin resistance gene at a ratio of 10:1. Cells resistant to G418 (0.4 mg/ml) were cloned. Cells were cultured in 2% low Ig fetal bovine serum to collect condition medium. Ecto-ErbB4 was purified by chromatography using HiTrap protein G beads (Amersham).

Immunoprecipitation and Western Blotting

Immunoprecipitation was carried out as previously described (Huang et al., 2000). Briefly, cell lysates (1 mg of protein) were incubated with indicated antibodies (1–2 μg) at 4°C for 1 hr with constant rocking in 1 ml of the modified RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.25% sodium-deoxycholate, 1 mM PMSF, 1 mM EDTA, 1 μg/ml aprotinin, leupeptin, and pepstatin protease inhibitors). Samples were then incubated at 4°C for 1 hr with agarose beads (1:1 slurry, 50 μl) conjugated with protein A (for rabbit antibodies) or G (for mouse antibodies). Bound proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane, which was blocked with TBS containing 5% nonfat dry milk and 0.05% Tween 20 for 1 hr. The membrane was then incubated overnight at 4°C with primary antibodies and developed by horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence system (Amersham Pharmacia).

In Situ Hybridization

In situ hybridization was performed essentially as previously described (Simmons et al., 1989), with minor modifications. Adult Sprague-Dawley rats were perfused for 20 min with 4% paraformaldehyde in 0.1 M sodium borate buffer (pH 9.5). Sagittal sections (30 μm) were cut on a sliding microtome and mounted on gelatin and poly-L-lysine-coated slides. Tissue sections were fixed for 30 min in 10% buffered formalin and washed in 50 mM KPBS prior to prehybridization. ErbB4 sequence #1009-1931 (accession # NM-021687), NRG1 type I/II sequence #345-845 (accession # NM-031588), and NRG1 type III sequence #555-1321 (accession #AF194438) were subcloned in pCRScript. Plasmids were digested with NotI, SpeI, and EcoRI, respectively, for the production of individual antisense RNAs using T7 RNA polymerase. Transcriptions were performed using 125 μCi ^{33}P-UTP (2000–4000 Ci/mmole, NEN). After hybridization, the sections were defatted in xylene, rinsed in 100% ethanol and then 95% ethanol, air dried, and dipped in NTB2 emulsion (Kodak) diluted 1:1 with water. The slides were exposed for 2–5 weeks and developed in Kodak D-19 developer. All images were captured with a Hamamatsu Orca ER CCD camera using dark-field microscopy on an Olympus BX-51 microscope at 1.25 × magnification.

Immunostaining

Immunostaining of rat cortical neurons (E17, DIV14) was performed as previously described (Huang et al., 2000). Briefly, neurons were fixed with 4% paraformaldehyde and 4% sucrose in PBS for 20 min, and permeabilized by incubation in PBS containing 1% BSA and 0.1% Triton X-100 for 30 min at room temperature. After washing, neurons were incubated in the buffer containing antibodies against phospho-ErbB4 (1:200), GAD65 (1:200), or both for 1 hr at room temperature. Brain sections (20 μm) were fixed with 10% formaldehyde and blocked in 5% BSA/1% normal goat serum (Ren et al., 2004). Sections were incubated overnight at 4°C in PBS containing rabbit anti-ErbB4 with or without anti-GAD65 or VGAT. Fluorochrome-conjugated secondary antibodies were used to visualize the immunoreactivity with a confocal microscope.

Statistical Analysis

Data were presented as mean ± SEM of three or more independent experiments. For multiple group comparisons, statistical differences were calculated by one-way ANOVA followed by Dunnett's test. For comparison of means from the same group of cells, Student's paired t test was used. mIPSCs were analyzed by the Kolmogorov-Smirnov (K-S) test. Values of $p < 0.05$ were considered significant.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/54/4/599/DC1/.

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